(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18252 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/US00/24639

(22) International Filing Date:
8 September 2000 (08.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/152,847	8 September 1999 (08.09.1999)	US
09/455,950	7 December 1999 (07.12.1999)	US

(71) Applicant: **EXACT LABORATORIES, INC.** [US/US]; 63 Great Road, Maynard, MA 01754 (US).

(72) Inventor: **SHUBER, Anthony, P.**; 11 Grant Street, Milford, MA 01757 (US).

(74) Agent: **CAMACHO, Jennifer, A.**; Testa, Hurwitz & Thibeault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).

(81) Designated States *(national)*: AU, CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/18252 A2

(54) Title: METHODS FOR DISEASE DETECTION

(57) Abstract: The present invention provides methods for detecting disease by analysis of a patient sample to determine the integrity of nucleic acids in the sample.

METHODS FOR DISEASE DETECTION

Background of the Invention

Many diseases are associated with genomic instability. That is, a disruption in genomic stability, such as a mutation, has been linked to the onset or progression of certain diseases. Accordingly, various aspects of genomic instability have been proposed as reliable markers for disease. For example, mutations in the BRCA genes have been proposed as markers for breast cancer, and mutations in the p53 cell cycle regulator gene have been associated with numerous cancers, especially colorectal cancer. It has been suggested that specific mutations might be a basis for molecular screening assays for the early stages of certain types of cancer. See, e.g., Sidransky, et al., Science, 256: 102-105 (1992).

The search for genomic disease markers has been especially intense in the area of cancer detection. Cancer is characterized by uncontrolled cell growth which can be associated with one or more genetic mutations. Such mutations can cause the affected cells to avoid cell death. For example, a mutation in a tumor suppressor gene can cause cells to avoid apoptosis - a type of cell death thought to be under direct genetic control. During apoptosis, cells lose their membranes, the cytoplasm condenses, and nuclear chromatin is split into oligonucleotide fragments of characteristically short length. In fact, those characteristic DNA cleavage patterns have been proposed as an assay for apoptosis.

Attempts have been made to identify and use nucleic acid markers that are indicative of cancer. However, even when such markers are found, using them to screen patient samples, especially heterogeneous samples, has proven unsuccessful either due to an inability to obtain sufficient sample material, or due to the low sensitivity that results from measuring only a single marker. Simply obtaining an adequate amount of human DNA from one type of heterogeneous sample, stool, has proven difficult. See Villa, et al., Gastroenterol., 110: 1346-1353 (1996) (reporting that only 44.7% of all stool specimens, and only 32.6% of stools from healthy individuals produced sufficient DNA for mutation analysis). Other reports in which adequate DNA has been obtained have reported low sensitivity in identifying a patient's disease status based upon a single

cancer-associated mutation. See Eguchi, et al., Cancer, 77: 1707-1710 (1996) (using a p53 mutation as a marker for cancer).

Investigators have attempted to analyze mutations in DNA of tumor cells shed into luminal areas, such as the colon, bile ducts, blood vessels and the like. Such attempts have only been successful when there is a known mutation and a relatively high concentration of cellular material has been found. See e.g., Mulcahy, et al., Ann. Oncol. 10 Suppl 4:114-117 (1999). No attempts have been made to correlate disease status with DNA integrity in shed cellular material.

Summary of the Invention

The present invention provides that the integrity of nucleic acids in biological samples comprising shed cellular material is an indicator of the disease status of the patient from whom the sample was obtained. According to the invention, certain tissue or body fluid samples, especially those described below, contain debris from cells that have been shed from surrounding organs or tissue. In healthy patients, such debris is the result of apoptosis as part of the normal cell cycle. Apoptosis reduces nucleic acid integrity, so that only small-fragment nucleic acids exist in exfoliated cellular debris in healthy individuals. To the contrary, in diseases such as cancer in which cell cycle mechanisms are destroyed or impaired, cellular debris comprises high-integrity nucleic acids (i.e., nucleic acids that have not been degraded by apoptosis). Thus, methods of the invention comprise using nucleic acid integrity as a measure of patient disease status. Integrity can be measured by any convenient means. Preferred means include the amount of nucleic acid in a sample, the length of nucleic acids in a sample, or the molecular weight of nucleic acids in a sample.

The invention provides methods for detecting disease in a patient based upon the integrity of patient nucleic acids present in a specimen or sample obtained from the patient. According to methods of the invention, a tissue or body fluid specimen containing sloughed cellular debris obtained from a patient having a disease contains an amount of intact nucleic acid that is greater than would be expected in such a specimen obtained from a healthy patient. Thus, a measure of intact nucleic acid in a patient sample is indicative of the overall disease status of the patient. As used herein, "intact" refers to nucleic acids that are longer than those expected to be present as a

result of apoptosis. The invention is equally applicable to human and to veterinary uses. Accordingly, "patient" as defined herein means humans or other animals.

A healthy patient generally produces cellular debris through normal apoptotic degradation, resulting in relatively short nucleic acid fragments in samples derived from luminal tissue and fluids. Patients having a disease generally produce cells and cellular debris, a proportion of which has avoided normal cell cycle regulation, resulting in relatively long, intact nucleic acid fragments. Without being held to theory, the present invention takes advantage of this and other insights concerning the ways in which cells respond to diseases, especially diseases associated with genetic abnormalities (either induced or inherited). As a result, it has been discovered that the disease status of a patient is determined by analysis of patient nucleic acids produced in specimens obtained from the patient. Most preferably, such specimens are those most likely to contain sloughed cellular debris. Such specimens include, but are not limited to, stool, blood serum or plasma, sputum, pus, colostrum, and others. In diseases, such as cancer, in which genomic instabilities or abnormalities have interfered with normal cell cycle regulation, specimens such as those identified above contain relatively intact nucleic acid fragments. The presence of such fragments is a general diagnostic screen for disease.

Accordingly, methods of the invention comprise screening a patient for disease by analysis of the integrity of nucleic acids in a tissue or body fluid specimen obtained from the patient. Preferred specimens include those comprising shed cells or cellular debris. Thus, highly-preferred specimens are those not containing an abundance of intact (non-exfoliated) cells. Such preferred specimens comprise stool, sputum, urine, bile, pancreatic juice, and blood serum or plasma, all of which contain shed cells or cellular debris. Methods of the invention are especially useful as screens for cancer. Cancer is a disease thought to be associated with genomic instabilities, and specifically with the loss of control over the normal cell cycle. Thus, tumor cells are typically intact and routinely are shed into, for example, stool, sputum, urine, bile, pancreatic juice, and blood. Such shed cells and cellular debris contain higher integrity nucleic acids compared to those found in specimens obtained from a healthy patient. There are numerous ways in which the integrity of nucleic acids in a patient specimen are measured as a screen for disease.

In a preferred embodiment, nucleic acid integrity is measured by the ability to amplify nucleic acids in a sample. Thus, a preferred method comprises conducting in a tissue or body fluid sample an amplification reaction using as a template a nucleic acid locus suspected to be in the sample. If the amount of amplification product (amplicon) is greater than the amount of amplicon expected to be present in a normal sample (e.g., one not having the disease being screened), the sample is determined to be positive. In some cases, the presence of any amplification product is sufficient to justify a positive screen for disease. It is preferable that, in the case of DNA, the amplification reaction is a polymerase chain reaction (PCR) or, in the case of RNA, that the amplification reaction is reverse transcriptase PCR. Primers are designed to amplify the locus or loci chosen for analysis. For purposes of the invention a "genomic locus" is any genetic element, including but not limited to a coding region of a gene, a non-coding nucleic acid region, a regulatory element of a gene, an intron or RNA. It is not required that the target genomic loci be associated with any specific disease, as an increase in amplifiable nucleic acid is itself diagnostic.

In one preferred embodiment, the presence of a single high molecular weight amplicon is a positive screen. Preferably, a fragment of about 1.3 Kb or greater is measured as an indicator of high integrity nucleic acids in the patient sample.

In a highly-preferred embodiment, a profile of amplification products across a range of nucleic acid fragments of different lengths is produced. In a preferred embodiment, a series of amplification reactions is conducted at a single genomic locus, each reaction being designed to amplify a fragment of unique length. If detectable amplicon is produced in each reaction, or in a number of reactions greater than expected in a sample obtained from a healthy patient, the sample is determined to be positive. For example, attempts are made to amplify fragments of 200 bp, 400 bp, 800 bp, 1.3 Kb, 1.8 Kb, and 2.4 Kb at the same genomic locus. In a sample obtained from a healthy individual (a "normal" sample), it would be expected that little or no amplification product is observed, especially when the longer portions of the locus are used as the template. To the contrary, at least some proportion of cells and cellular debris in a sample obtained from a diseased patient will contain intact fragments.

In another embodiment, a profile of amplification products across a range of nucleic acid fragments of different lengths is produced by a series of amplification reactions conducted on a series of different genomic loci, each reaction being designed

to amplify a fragment of unique length. If detectable amplicon is produced in each reaction, or in a number of reactions greater than expected in a sample obtained from a patient not having the disease being screened, the sample is determined to be positive.

According to methods of the invention, normal samples do not produce significant amounts of detectable amplicon at any length significantly greater than the typical apoptotic fragment (about 175 bp). Accordingly, whether primers are spaced to amplify fragments of only one length at a given genomic locus, or whether a series of amplifications at the locus are conducted, differences are readily observable between normal and diseased samples.

As detailed below, methods of the invention are useful to detect disease, preferably cancer or precancer, in biological samples comprising shed cells or cellular debris. For example, the presence in a patient stool sample of amounts of nucleic acid, preferably DNA, above a predetermined threshold for healthy patients is indicative that the patient has cancer. Follow-up analysis is used to determine where the disease resides. However, the general disease screen is effective independent of the locus of the disease and the specimen taken for analysis. Thus, while the analysis of nucleic acids in stool is predictive of disease generally, it does not necessarily indicate that the disease is of gastrointestinal origin. However, follow-up screening based, for example, on mutational analysis, is adequate to identify the locus of disease. Numerous mutational analyses are known in the art and include, for example, U.S. Patent No. 5,670,325, incorporated by reference herein.

In an alternative embodiment, screening of patient samples by detecting amounts of nucleic acid in the sample is combined with an assay for apoptotic cell activity. Such assays may be combined with detecting amounts of nucleic acid in a patient sample as a screen for disease status. A positive screen is one that produces both: (1) an amount of nucleic acid that is greater than the amount expected to be present in a normal sample (e.g., one not having the disease being screened), and (2) an amount of apoptotic cell activity that is less than that expected to be present in a normal sample. In a highly preferred embodiment, methods of the invention comprise analyzing a plurality of genomic loci to determine an amount of amplifiable nucleic acid present at each locus. Analysis across multiple loci using methods of the invention may increase the sensitivity of the screening assay.

As will be exemplified in detail below, methods of the invention comprise screening a biological sample for an abnormality in a nucleic acid by conducting an amplification reaction using as a template a nucleic acid suspected or expected to be in the sample; determining an amount of amplification product obtained; comparing the amount of amplicon obtained to a standard amount of amplification product; and identifying a sample as having an abnormality in a nucleic acid if the amount of amplification product differs from the standard amount. In a preferred embodiment, a standard amount of amplification product is determined by amplification of a locus, or portion thereof, being screened (e.g., an intact, wild-type nucleic acid) in a known normal sample (one obtained from an individual known not to have the disease being screened). Also in preferred embodiments, a standard amount is determined by reference to the art. In certain embodiments of the invention, the standard amount is essentially no detectable amplicon due to the lack of high-integrity nucleic acids in the sample. Accordingly, any detectable amplicon in a patient sample is indicative of a positive screen. That is the case especially when a large (e.g., 1.8 Kb or 2.4 Kb) fragment is being screened. Finally, the standard amount can be a molecular weight marker on, for example, an electrophoretic gel.

In a preferred embodiment of the invention, the sample is prepared from a specimen selected from the group consisting of stool, sputum, blood, urine, cerebrospinal fluid, seminal fluid, saliva, breast nipple aspirate, and biopsy tissue. However, any tissue or body fluid specimen may be used according to methods of the invention. Especially preferred are samples of luminal fluid because such samples are generally free of intact, healthy cells. Such samples include blood, urine, bile, pancreatic juice, stool, sputum, pus, and the like.

Also in a preferred embodiment, the nucleic acid or nucleic acids being interrogated is (are) DNA. In a more particular embodiment, the nucleic acid being analyzed is selected from a coding region of a gene, or portion thereof, a noncoding nucleic acid region, or portion thereof, a regulatory element of a gene or a portion thereof, and an unidentified fragment of genomic DNA. Also in a preferred embodiment, the nucleic acid being interrogated is RNA. As is appreciated by the skilled artisan, any genomic locus is amenable to screening according to the invention. The particular locus or loci chosen for analysis depends, in part, on the disease being screened, and the convenience of the investigator. It is not necessary that the locus or loci chosen for

analysis be correlated with any specific disease because methods of the invention contemplate measuring either the total nucleic acid in a sample or amplifiable nucleic acid in a sample as an indicator of overall disease status or the presence and/or extent of apoptosis in the sample. However, disease-associated loci (those in which a mutation is indicative, causative, or otherwise evidence of a disease) can be used. Preferred disease-associated loci include p53, apc, MSH-2, dcc, scr, c-myc, B-catnenin, mlh-1, pms-1, pms-2, pol-delta, and bax.

The amount of amplification product may be determined by any suitable or convenient means. Preferably, the amount of amplification product is determined by gel electrophoresis. Labels, such as fluorescent or radioactive labels, may be used. The amounts of amplification product produced may be compared to standard amounts by any suitable or convenient means, including, but not limited to visual comparison, machine-driven optical comparison, densitometry, mass spectroscopy, hybrid capture, and other known means. The amplification reaction itself can be any means for amplifying nucleic acid, including, but not limited to PCR, RT-PCR, OLA, rolling circle, single base extension, and others known in the art. The amplification product can also be measured by signal amplification techniques, such as branch chain amplification (Chiron). Methods of the invention are useful with any platform for the identification, amplification, sequencing, or other manipulation of nucleic acids. For example, methods of the invention can be applied to ligase chain reaction, strand displacement (Becton-Dickinson), and others.

Also in a preferred embodiment of the invention, a series of amplification reactions is conducted on a single genomic locus. Each amplification reaction in the series is designed to amplify a fragment of a different length. In a preferred embodiment, the target fragment lengths are 200 bp, 400 bp, 800 bp, 1.3 Kb, 1.8 Kb, and 2.4 Kb. Primers for amplification are designed according to knowledge in the art in order to amplify template, if present, of the desired length at the desired locus. A positive screen is one that produces amplicon in at least one, and preferably at least two of the series of amplification reactions. As noted above, a normal sample which has undergone or which is undergoing apoptosis typically contains little or no fragments of significant length. Thus, a series of amplification reactions targeting fragments from about 200 bp to about 2.4 Kb and longer reveals samples that contain nucleic acids that have avoided apoptosis as evidenced by the amplification of large fragments.

Preferred methods of the invention also comprise conducting amplification reactions on a series of different genomic loci. Preferably, from about 2 to about 7 loci are used. However, the precise number of interrogated loci is determined by the individual investigator based upon the disease to be detected or based upon convenience. According to methods of the invention, primers are designed to amplify nucleic acid (preferably DNA) at each of the chosen loci. A sample in which at least one locus, preferably at least two loci, and most preferably at least three loci produce detectable amplification product is considered a positive sample. The lengths of fragments to be amplified in this assay may be varied, but are preferably at least about 180 bp each in length. It is not necessary that the same length fragments be amplified at each of the chosen loci.

Methods of the invention also comprise conducting a series of amplification reactions at a series of different genomic loci. Each amplification reaction in the series is designed to amplify a fragment of a different length. Preferably, from about 2 to about 7 amplification reactions on about 2 to about 7 loci are used. However, the precise number of interrogated loci is determined by the individual investigator based upon the disease to be detected or based upon convenience. In a preferred embodiment, the target fragment lengths are 200 bp, 400 bp, 800 bp, 1.3 Kb, 1.8 Kb, and 2.4 Kb. Primers for amplification are designed according to knowledge in the art in order to amplify template if present. It is preferred, but not necessary, that the same length fragments be amplified at each of the chosen loci. A positive screen is one that produces amplicon in at least one, and preferably at least two of the series of amplification reactions and in which at least one locus, preferably at least two loci, and most preferably at least three loci produce detectable amplification product. As noted above, a normal sample which has undergone or which is undergoing apoptosis typically contains little or no fragments of significant length. Thus, a series of amplification reactions targeting fragments from about 200 bp to about 2.4 Kb and longer reveals samples that contain nucleic acids that have avoided apoptosis as evidenced by the amplification of large fragments.

Methods of the invention may also be used to assess the integrity of DNA in a biological sample. Such methods comprise conducting an amplification reaction using at least two loci suspected to be in the sample as templates; determining which loci produce detectable amplicon; and assessing the integrity of DNA in the sample as a

function of the number of loci producing amplicon. The integrity of DNA in the sample is high when amplicon is produced in one or more of the amplification reactions. This method is especially useful for determining whether a heterogeneous sample has sufficient nucleic acid for measurement. Accordingly, such methods are used to screen or to "qualify" samples for further analysis (e.g., genetic, biochemical, cytological, or other analyses).

Methods of the invention may also be used to assess fetal abnormalities by conducting amplification reactions on nucleic acids in maternal blood. Just as described above, the ability to amplify significant amounts of nucleic acid is an indicator of a genomic instability. A baseline for comparison of the extent of nucleic acid amplification can be amounts of nucleic acids from known normal samples. The amount of amplification obtained from fetal samples is placed on a continuum, and the investigator must analyze any given sample in terms of the amount of fetal nucleic acid produced in various disease states and in normal samples.

Methods of the invention are useful as diagnostic screening methods. Often it is desirable to perform follow-up testing on a patient in order to confirm a suspected disease state. Such follow-up procedures are determined based upon the disease state being interrogated. For example, a colonoscopy may be suggested in a case in which a stool sample is positively screened according to methods of the invention. Such follow-up procedures are contemplated herein as part of the invention.

Methods of the invention are useful as screens for a wide range of disease states. In addition to colon cancers and adenomas, methods of the invention are useful to screen for other diseases, for example, as screens for lymphomas, or stomach, lung, liver, pancreas, prostate, kidney, testicular, bladder, uterus, or ovarian cancers or adenomas. In addition to cancer, methods of the invention are useful, for example, as screens for diseases such as inflammatory bowel syndrome, inflammatory bowel disease, Crohn's disease, and others in which a genomic instability is thought to play a role. Methods of the invention are especially useful as screens for any disease that impairs the proper function of the gastrointestinal system; most especially diseases of the colon. Methods of the invention are also useful to screen for apoptosis in a cellular sample. The profile of amplifiable DNA in a sample is correlated with proteins that have been associated with disease. For example up regulation of the apoptosis protein,

survivin, is correlated with increased amounts of amplifiable DNA, as is the Ras oncogene, as well as other oncogenes and their gene products.

Methods of the invention are also useful as assays for apoptosis. The presence of high-integrity fragments or large quantities of nucleic acids in a sample indicates that the sample was derived from cells that did not proceed through apoptosis. The absence of such fragments or quantities indicates that cells that contributed to the sample did undergo apoptosis. Accordingly, an apoptotic activity assay of the invention, either alone or in combination with other assays for genomic instability, are useful as screens for disease.

Finally, methods of the invention can be carried out by hybrid capture. For example, hybrid capture and subsequent analysis of the captured fragments can be used to determine the nucleic acid integrity of a sample.

The invention also provides a profile of nucleic acid fragments indicative of disease. A preferred profile is obtained through methods described above. Preferred profiles comprise nucleic acids having between about 200 bp and about 2.4 Kb obtained in a patient sample comprising cellular debris according to methods described herein. A highly preferred profile contains at least one nucleic acid of at least 1.3 Kb.

Other objects and advantages of the invention are apparent upon consideration of the following drawings and detailed description thereof.

Description of the Drawings

Figure 1 is a gel photograph showing results of amplification of K-ras (exon 1) DNA isolated from stool using forward and reverse primers spaced about 200 bp apart. The band intensity relates to the amount of 200 bp product or greater in the sample. Lanes 1-4 are results from patients with cancer or adenoma, lane 5 is a positive control, lanes 6-10 are from patients who did not have cancer or adenoma, lanes 11-12 are negative controls, and lanes 13-18 are standards at the approximate molecular weight indicated in the figure. Amplifications were graded A through C, A being the most intense band, C being the least.

Figures 2-4 are gel photographs showing results of amplification of apc (exon 15) DNA isolated from stool using forward and reverse primers spaced about 200 bp apart. The band intensity relates to the amount of 200 bp product or greater in the sample. Lanes 1-4 are results from patients with cancer or adenoma, lane 5 is a positive control,

lanes 6-10 are from patients who did not have cancer or adenoma, lanes 11-12 are negative controls, and lanes 13-18 are standards at the approximate molecular weight indicated in the figure. Amplifications were graded A through C, A being the most intense band, C being the least.

Figure 5 is a gel photograph showing results of amplification of p53 (exon 5) DNA isolated from stool using forward and reverse primers spaced about 200 bp apart. The band intensity relates to the amount of 200 bp product or greater in the sample. Lanes 1-4 are results from patients with cancer or adenoma, lane 5 is a positive control, lanes 6-10 are from patients who did not have cancer or adenoma, lanes 11-12 are negative controls, and lanes 13-18 are standards at the approximate molecular weight indicated in the figure. Amplifications were graded A through C, A being the most intense band, C being the least.

Figure 6 is a gel photograph showing results of amplification of p53 (exon 7) DNA isolated from stool using forward and reverse primers spaced about 200 bp apart. The band intensity relates to the amount of 200 bp product or greater in the sample. Lanes 1-4 are results from patients with cancer or adenoma, lane 5 is a positive control, lanes 6-10 are from patients who did not have cancer or adenoma, lanes 11-12 are negative controls, and lanes 13-18 are standards at the approximate molecular weight indicated in the figure. Amplifications were graded A through C, A being the most intense band, C being the least.

Figure 7 is a gel photograph showing results of amplification of p53 (exon 8) DNA isolated from stool using forward and reverse primers spaced about 200 bp apart. The band intensity relates to the amount of 200 bp product or greater in the sample. Lanes 1-4 are results from patients with cancer or adenoma, lane 5 is a positive control, lanes 6-10 are from patients who did not have cancer or adenoma, lanes 11-12 are negative controls, and lanes 13-18 are standards at the approximate molecular weight indicated in the figure. Amplifications were graded A through C, A being the most intense band, C being the least.

Figure 9-10 are gel photographs of results of amplification of DNA from stool samples using forward and reverse primers spaced approximately 1.8 Kb apart. The band intensity shows the amount of 1.8 Kb or greater product. Lanes 1, 8, and 9 are negative controls, lanes 2, 3, and 5 are results from patients with cancer or adenoma,

lanes 4, 6, and 7 are results from patients who did not have cancer or adenoma, and lanes 10-14 are molecular weight standards.

Figures 11 A and B are gel photographs of results of amplification of DNA in stool from a total of 30 patients and controls. The band intensity relates to the amount of amplifiable DNA in the sample. Lanes N are negative controls, lanes 1, 3, 11, and 18 are results from patients which are indicative of the presence off cancer or adenoma, lanes 2, 4, 5-10, 12-17, and 19-30 are results from patients which are indicative of the absence of cancer or adenoma. The remaining lanes are markers or standards.

Figure 12 shows a schematic representation of the placement of the primers for amplification in a method of the present invention. In this method, a single forward primer, F_1, is used in conjunction with a series of reverse primers, R_1 to R_6, chosen to amplify progressively longer portions of the target.

Figure 13 shows a schematic representation of the placement of the primers for amplification in a method of the present invention. In this method, a series of forward and reverse primer pairs, (F_1 , R_1) to (F_3 , R_3), are chosen to amplify portions of the target spaced at intervals along the target.

Detailed Description of the Invention

The invention provides methods for the analysis of biological samples. Methods of the invention provide diagnostically-relevant information based upon the integrity of nucleic acids in a biological sample. Normal biological samples (those not having indicia of the disease being screened), especially those comprising luminal tissue and/or fluid, typically comprise a majority of short-fragment, low-integrity nucleic acids (especially DNA) which are the result of degradation by apoptosis. When a mutation has caused genomic instability, the normal cell cycle may be disrupted and apoptotic degradation may not occur at the rate expected in a normal sample. Methods of the invention screen for such disruptions.

Accordingly, preferred methods of the invention comprise determining an amount of amplifiable nucleic acid in a biological sample, and determining whether that amount is consistent with an amount expected in a normal sample. In many biological samples, especially heterogeneous samples, there may be no detectable amplification product. That is especially true when longer fragments are used as templates for amplification. Generally, the probability that any given set of PCR primers will amplify a DNA fragment having a length exceeding the primer distance is expressed as

% of Fragments Amplified = (FL-PD)/(FL+PD)

wherein FL is fragment length (in base pairs) and PD is primer distance (in base pairs). This equation assumes that sample DNA fragment lengths are uniformly distributed (i.e., there is no favored locus at which breaks occur).

In a preferred embodiment, methods of the invention comprise amplifying sequences of different length in a sample, if present, in order to generate a profile of amplification products indicative of disease or the propensity for disease. In a preferred method, a sample is exposed to a set of PCR primers comprising a single forward primer, which may be a capture probe used to capture target fragments, and a plurality of downstream reverse primers which hybridize to portions of a contiguous sequence (if present) in the sample. Amplifications using these primers will result in a series of amplification products, each having a different length, if the contiguous target sequence is present in the sample. The length of the amplification products are determined by the spacings between the forward primer and each of the downstream reverse primers. An example is shown in Figure 12, which is a schematic representation showing placement of the primers for amplification.

If the target sequence, or a portion of it, is present in the sample, amplification will result in a series of fragments the length of which is dictated by the spacing of the primers. According to the principles adduced above, a sample from a diseased patient will produce a profile of amplification products in the assay described above that differs from the profile obtained from a sample containing the smaller fragments expected to be produced as a result of normal apoptosis. In a preferred embodiment, the forward primer is designed to hybridize about 200 bp upstream of the first reverse primer, and about 2.3 Kb upstream of the last reverse primer. Other reverse primers are designed to hybridize at various locations between the first and last reverse primers. Preferred intervals between the forward primer and the various reverse primers are 200 bp (F_1-R_1), 400 bp (F_1-R_2), 800 bp (F_1-R_3), 1.3 Kb, (F_1-R_4), 1.8 Kb (F_1-R_5), and 2.3 Kb (F_1-R_6). The number and spacing of reverse primers is chosen at the convenience of the skilled artisan.

Also in a preferred embodiment, a hybrid capture probe is used to anchor a target sequence, preferably on a solid support (e.g., beads). A plurality of probes are then placed at various distances downstream of the capture probe. Those probes can be pairs of forward and reverse primers as discussed above, or they can be signal

amplification probes, such as those used in Ligase Chain Reaction (LCR), and others used in the identification of sequences. The plurality of probes hybridize along the length of a target fragment if the target is present in the sample. Thus, by interrogating samples for the presence of the probes, one can determine the integrity of sequences present in the sample. This can be done in numerous ways, including, but not limited to, hybrid capture, PCR, LCR, strand displacement, branched chain, or other assays known in the are that incorporate hybrid probes or primers in order to identify or quantitate sequence. A sample containing intact (high integrity) nucleic acids represents a positive screen according to the invention. In one embodiment, sample is placed into wells (e.g., on a 96 well plate) containing support-bound capture probe. The capture probe immobilizes a target sequence, if present in the sample. Probes that hybridize to sequence downstream of the capture probe (downstream probes) are placed into each well, such that each downstream probe is spaced a unique distance apart from the common capture probe, and each well contains only one type of downstream probe. Signal is then generated by, for example, amplification, or by standard ELISA procedure followed by amplification, or by LCR, or other methods mentioned above. The presence of signal in each well indicates the presence of sequence of at least the length between the capture probe and the downstream probe. In an alternative embodiment, each well receives multiple different downstream probes, which may be distinctly labeled, and the presence of label(s) is correlated with the length of sequence presence in the sample.

A sample from a patient having, for example, cancer will produce amplicon between most or all of the primer pairs (depending, *inter alia,* on the length of the target fragments, on the spacing of the primers, and where on the target the primers are spaced). Such a profile represents a positive screen for disease or the propensity for disease. A sample from a patient who does not have indicia of disease results in little or no amplification product in the assay described above. In a negative screen there may be amplification of small (e.g., 200 bp) fragments but there should be no amplification of larger fragments (i.e., fragments resulting from amplification between the forward primer and spaced-apart reverse primers). In cancer diagnostics, the target fragment may optionally be an oncogene, a tumor suppressor, or any other marker associated with cancer. However, it is not necessary to use cancer-associated markers in methods of the invention, as such methods are based on the general recognition that samples

indicative of disease contain a greater amount of intact nucleic acids and a greater amount of long fragment nucleic acids. Accordingly, any convenient target nucleic acid locus may be used in the methods of the invention.

The amplification reactions described above may be conducted according to any suitable or convenient protocol and the fragment size of the resulting amplification products (if any) may be determined by any suitable or convenient means.

In an alternative embodiment, methods of the invention comprise conducting a series of amplification reactions on a contiguous nucleic acid target fragment, each application reaction comprising one forward primer and one reverse primer, such that pairs of forward and reverse primers are spaced at intervals on a contiguous fragment suspected to be in the sample. An example of this arrangement is shown in Figure 13. Preferably, the spacings between each forward and reverse primer pair are equivalent. In a positive screen, the assay described above will result in a series of same-size fragments for most if not all of the primer pairs. Such an array of amplification products evidences a contiguous target sequence indicative of disease (see above). A sample from a disease-free patient should produce little or no amplification product, but in any case will not produce the contiguous array of amplification products expected from a sample containing a relatively intact diagnostic target sequence.

Each of the methods described above are based upon the principle that an intact nucleic acid, or a segment of an intact nucleic acid, in a sample is diagnostic. Thus, variations on the methods described above are contemplated. Such variations include the placement of primers, the number of primers used, the target sequence, the method for identifying sequences, and others. For example, in the method depicted in Figure 13, and described above, it is not necessary that the numbers of forward and reverse primers be equal. A forward primer may, for example, be used to amplify fragments between two reverse primers. Other variations in primer pair placement are within the skill in the art, as are details of the amplification reactions to be conducted. Finally, as represented in Figures 12 and 13, capture probes may be used in methods of the invention in order to isolate a chosen target sequence.

The following examples provide further details of methods according to the invention. For purposes of exemplification, the following examples provide details of the use of the method if the present invention in colon cancer detection. Accordingly, while

exemplified in the following manner, the invention is not so limited and the skilled artisan will appreciate its wide range of application upon consideration thereof.

Exemplary Method for the Detection of Colon Cancer

The following example relates to screening for colon cancer in voided stool samples. Based upon the principles upon which the invention is based (see above), the same analysis can be performed on other samples, such as those mentioned above, with the same results as shown herein.

For the analysis of stool samples, preferred methods of the invention comprise obtaining at least a cross-sectional or circumferential portion of a voided stool as taught in U.S. patent number 5,741,650, and co-pending, co-owned U.S. patent application serial number 09/059,718, both of which are incorporated by reference herein. While a cross-sectional or circumferential portion of stool is desirable, methods provided herein are conducted on random samples obtained from voided stool, which include smears or scrapings. Once obtained, the stool specimen is homogenized. A preferable buffer for homogenization is one that contains at least 16 mM ethylenediaminetetraacetic acid (EDTA). However, as taught in co-pending, co-owned U.S. patent application serial number 60/122,177, incorporated by reference herein, it has been discovered that the use of at least 150 mM EDTA greatly improves the yield of nucleic acid from stool. Thus, a preferred buffer for stool homogenization comprises phosphate buffered saline, 20-100 mM NaCl or KCl, at least 150 mM EDTA, and optionally a detergent (such as SDS) and a proteinase (e.g., proteinase K).

After homogenization, nucleic acid is preferably isolated from the stool sample. Isolation or extraction of nucleic acid is not required in all methods of the invention, as certain detection techniques can be adequately performed in homogenized stool without isolation of nucleic acids. In a preferred embodiment, however, homogenized stool is spun to create a supernatant containing nucleic acids, proteins, lipids, and other cellular debris. The supernatant is treated with a detergent and proteinase to degrade protein, and the nucleic acid is phenol-chloroform extracted. The extracted nucleic acids are then precipitated with alcohol. Other techniques can be used to isolate nucleic acid from the sample. Such techniques include hybrid capture, and amplification directly from the homogenized stool. Nucleic acids can be purified and/or isolated to the extent required by the screening assay to be employed. Total DNA is isolated using techniques known in the art.

Screening Assay Protocol

The size of human DNA fragments obtained above can be determined by numerous means. For example, human DNA can be separated using gel electrophoresis. A 3% agarose gel is prepared using techniques known in the art. See Ausubel et. al., Short Protocols in Molecular Biology, John Wiley & Sones, 1195, pgs. 2-23-2-24, incorporated by reference herein. The size of human DNA fragments is then determined by comparison to known standards. Fragments greater than about 200 bp provide a positive screen. While a diagnosis can be made on the basis of the screen alone, patients presenting a positive screen are preferably advised to seek follow-up testing to render a confirmed diagnosis.

A preferred means for determining human DNA fragment length uses PCR. Methods for implementing PCR are well-known. In the present invention, human DNA fragments are amplified using human-specific primers. Amplicon of greater than about 200 bp produced by PCR represents a positive screen. Other amplification reactions and modifications of PCR, such as ligase chain reaction, reverse-phase PCR, Q-PCR, and others may be used to produce detectable levels of amplicon. Amplicon may be detected by coupling to a reporter (e.g. fluorescence, radioisotopes, and the like), by sequencing, by gel electrophoresis, by mass spectrometry, or by any other means known in the art, as long as the length, weight, or other characteristic of the amplicons identifies them by size.

Examples

Experiments were conducted to determine whether characteristics of amplifiable DNA in stool were predictive of cancer or precancer in patients from whom stools samples were obtained. In the first experiment, the amount of amplifiable DNA was measured in each of several stool samples using PCR amplification to detect DNA fragments in the sample of at least 200 base pairs in length. The second experiment determined the amount of long fragments (greater than 200 base pair) in the same samples, and then determined ratios of long product to short product. The third experiment determined a profile of amplification products with nucleic acid fragment lengths of 200 bp, 400 bp, 800 bp, 1.3 Kb, 1.8 Kb and 2.4 Kb. The fourth and fifth experiments were clinical studies correlating the integrity of nucleic acids in patient stool samples with overall patient disease status.

EXAMPLE 1

Stool samples were collected from 9 patients who presented with symptoms or a medical history that indicated that a colonoscopy should be performed. Each stool sample was frozen. Immediately after providing a stool sample, each patient was given a colonoscopy in order to determine the patient's disease status. Based upon the colonoscopy results, and subsequent histological analysis of biopsy samples taken during colonoscopy, individuals were placed into one of two groups: normal or abnormal. The abnormal group consisted of patients with cancer or with an adenoma of at least 1 cm in diameter. Based upon these results, 4 of the 9 patients were placed into the abnormal group.

The samples were screened by hybrid capturing human DNA, and determining the amount of amplifiable DNA having at least 200 base pairs. Each frozen stool specimen, weighing from 7-33 grams, was thawed and homogenized in 500 mM Tris, 16 mM EDTA, and 10 mM NaCl, pH 9.0 at a volume, to mass ratio of 3:1. Samples were then rehomogenized in the same buffer to a final volume-to-mass ratio of 20:1, and spun in glass macro beads at 2356 xg. The supernatant was collected and treated with SDS and proteinase k. The DNA was then phenol-chloroform extracted and precipitated with alcohol. The precipitate was suspended in 10 mM Tris and 1 mM EDTA (1 x TE), pH 7.4. Finally, the DNA was treated with Rnase.

Human DNA was isolated from the precipitate by sequence-specific hybrid capture. Biotynilated probes against portions of the p53, K-ras, and apc genes were used.

The K-ras probe was 5'GTGGAGTATTTGATAGTGTATTAACCTTATGTGTGAC 3' (SEQ ID NO: 1).

There were two apc probes: apc-1309 was 5'TTCCAGCAGTGTCACAGCACCCTAGAACCAAATCCAG 3' (SEQ ID NO: 2), and apc-1378 was 5'CAGATAGCCCTGGACAAACAATGCCACGAAGCAGAAG 3' (SEQ ID NO: 3).

There were four probes against p53, the first (hybridizing to a portion of exon 5) was 5'TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGG3' (SEQ ID NO:4), the second (hybridizing to a portion of exon 7) was 5'ATTTCTTCCATACTACTACCCATCGACCTCTCATC3' (SEQ ID NO: 5), the third, also hybridizing to a portion of exon 7 was

5'ATGAGGCCAGTGCGCCTTGGGGAGACCTGTGGCAAGC3' (SEQ ID NO: 6); and finally, a probe against exon 8 had the sequence 5'GAAAGGACAAGGGTGGTTGGGAGTAGATGGAGCCTGG3' (SEQ ID NO: 7).

A 10 μl aliquot of each probe (20 pmol/capture) was added to a suspension containing 300 μl DNA in the presence of 310 μl 6M GITC buffer for 2 hours at room temperature. Hybrid complexes were isolated using streptavidin-coated beads (Dynal). After washing, probe-bead complexes were suspended at 25° C for 1 hour in 0.1x TE buffer, pH7.4. The suspension was then heated for 4 minutes at 85° C, and the beads were removed.

Captured DNA was then amplified using PCR, essentially as described in U.S. Patent No. 4,683,202, incorporated by reference herein. Each sample was amplified using forward and reverse primers through 7 loci (Kras, exon 1, APC exon 15 (3 separate loci), p53, exon 5, p53, exon 7, and p53, exon 8) in duplicate (for a total of 14 amplifications for each locus). Seven separate PCRs (40 cycles each) were run in duplicate using primers directed to detect fragments in the sample having 200 base pairs or more. Amplified DNA was placed on a 4% Nusieve (FMC Biochemical) gel (3% Nusieve, 1% agarose), and stained with ethidium bromide (0.5 μg/ml). The resulting amplified DNA was graded based upon the relative intensity of the stained gels. The results are shown in Figures 1-7. Each Figure represents the results for all 9 patients (including standards) for the seven different loci that were amplified. As shown in the Figures, each sample from a patient with cancer or adenoma was detected as a band having significantly greater intensity than the bands associated with samples from patients who did not have cancer or precancer. All four cancer/adenoma patients identified using colonoscopy were correctly identified by determining the amount of amplifiable DNA 200 base pairs or greater in length. As shown in Figures 1-7, the results were the same regardless of which locus was amplified. Accordingly, the amount of 200 bp or greater DNA in a sample was predictive of patient disease status.

EXAMPLE 2

An experiment was conducted that was essentially identical to the one described above in Example 1, but forward and reverse primers were placed such that fragments of about 1.8 Kb and above were amplified.

DNA was prepared as described above. Forward and reverse primers were spaced so as to hybridize approximately 1.8 Kb apart on three different loci (Kras, exon 1, APC, exon 15, and p53 exon 5). Thirty-three rounds of amplification were performed, and the resulting DNA was placed on a 3% agarose gel. The results are shown in Figures 8-10. As shown in the Figures (which show results from three separate experiments to amplify and detect "long" product), samples from individuals having cancer or precancer produced large amounts of high-molecular weight (in this case 1.8 Kb and above) DNA; whereas samples from patients who did not have cancer or precancer produced no DNA in the range of about 1.8 Kb and higher. Thus, the presence of high-molecular weight DNA was indicative of the disease status of the patient.

EXAMPLE 3

An experiment was conducted to determine the molecular weight profile of DNA from samples collected and prepared as part of a blind study on 30 patients who presented at the Mayo Clinic with suspected gastrointestinal disorders. Stool samples were obtained, and DNA was isolated as described above.

Prior to amplification, DNA was isolated from the samples by hybrid capture. Biotynilated probes against portions of the BRCA1, BRCA2, p53, APC genes were used.

The BRCA1 probe was
5'GATTCTGAAGAACCAACTTTGTCCTTAACTAGCTCTT3' (SEQ ID NO: 8).

The BRCA2 probe was
5'CTAAGTTTGAATCCATGCTTTGCTCTTCTTGATTATT3' (SEQ ID NO 9).

The APC1 probe was
5'CAGATAGCCCTGGACAAACCATGCCACCAAGCAGAAG3' (SEQ ID NO 10).

The p53 probe, hybridizing to a portion of exon 5, was
5'TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGG3' (SEQ ID NO:4).

The APC2 probe was
5'GAAGTTCCTGGATTTTCTGTTGCTGGATGGTAGTTGC3' (SEQ ID NO 11).

A 300 μl aliquot of sample was placed in 300 μl of 6 M guanidine isothiocyanate buffer with 10 μl of each capture probe, and incubated overnight at 25 C. Captured DNA was isolated using 100 μl capture beads incubated for one hour at room

temperature. The DNA was eluted off the beads and PCR amplified under standard PCR conditions.

According to methods of the invention, amplification reactions were conducted using forward and reverse primers through the 5 loci for each sample. Forward and reverse primers were spaced to amplify fragments of 200 bp, 400 bp, 800 bp, 1.3 Kb, 1.8 Kb, and 2.4 Kb. Each of 30 PCR reactions was run for 36 cycles. Amplicon was run on a 3% Seakeam gel, and stained with ethidium bromide. The results are shown in Figures 11A and 11B. Each figure represents the results for 15 of the 30 patients.

As shown in those figures, patients with cancer or adenoma have an increased yield of amplifiable DNA. That is especially true at the 1.8 Kb level and above. Thus, patients with cancer or adenoma not only produce more amplifiable DNA in their stool, but also produce larger DNA fragments than are produced in the stool of patients who do not have cancer. Thus, both an increased yield of amplifiable DNA and the presence of high molecular weight DNA, especially that at 1.8 Kb and above, were indicative of patient disease status.

EXAMPLE 4

In this example, methods of the invention were correlated with clinical outcome in numerous patients who had a colorectal adenoma or colorectal cancer as diagnosed using colonoscopy, and 79 patients who were diagnosed as not having colorectal cancer or adenoma. A stool sample was obtained from each of these patients and prepared as described above. Fragments of the 5 different loci referred to above were amplified using primers spaced 200, 400, 800, 1300, 1800, and 2400 base pairs apart using the protocol described above in Example 3. Each amplification was scored such that successful amplification of a fragment received a score of 1, and no amplification received a score of 0. Since five loci were interrogated using 6 primer pairs each, the maximum score was 30 (successful amplification of all 6 fragments at all five loci). The cutoff for a positive screen was set at 21. The results are shown below.

Table 1
Normals

Patient No.	Age	Score
P-178	64	19
P-185	50	18
P-033	56	16
P-177	67	14
P-055	75	13
P-029	70	12
P-079	63	12
P-066	72	11
P-027	65	10
P-054	72	9
P-158	59	9
P-043	56	8
P-009	73	7
P-030	86	2
P-032	51	1
P-068	58	1
P-187	63	1
P-018	68	0
P-186	61	17
P-135	67	14
P-120	75	13
P-179	76	9
P-057	56	7
P-143	65	6
P-136	58	1
P-012	75	0

Table 2
Adenomas

Patient No.	Age	Score
P-003		29
P-001		23
P-045		22
P-162		21
P-163		16
P-088		15
P-050		13
P-060		11
P-061		11
P1058		10
P-075		10
P-077		8
P-024		7
P-056		7
P-067		7
P-025		6
P-080		4
P-123		4
P-048		3
P-040		2
P-006		1
P-004		0
P-015		0
P-083		0
P-047		
P-129		

Table 3
Carcinomas

Patient No.	Age	Score
P-064		30
P-103		30
P-104		30
P-108		30
P-101		29
P-102		29
P-099		28
P-107		28
P-110		26
P-098		25
P-134		24
P-062		23
P-090		23
P-095		23
P-093		22
P-100		21
P-122		18
P-084		15
P-109		15
P-118		10
P-138		10
P-091		8
P-096		8
P-053		7
P-119		6
P-117		5
P-105		0
P-097		

As shown above, methods of the invention are effective in screening for the presence of colorectal cancer and adenoma.

EXAMPLE 5

In this example, methods of the invention were used to detect non-colonic cancers in 28 patients.

A stool sample was obtained from each of the 28 patients. The sample was prepared as described above. Fragments of the 5 different loci referred to above were amplified using primers spaced 200, 400, 800, 1300, 1800, and 2400 base pairs apart using the protocol described above in Example 3. Each amplification was scored such that successful amplification of a fragment received a score of 1, and no amplification received a score of 0. Since five loci were interrogated using 6 primer pairs each, the maximum obtainable score was 30 (successful amplification of all 6 fragments at all five loci). A score of 21 was used as a cutoff between diseased and non-diseased patients. The results are shown below.

Table 4
Supercolonic Cancers

Patient No.	Supercolonic Cancer	Age	Score
P-145	Pancreas	68	30
P-164	Lung CA	68	30
P-166	Bile Duct	52	30
P-189	Bile Duct	43	30
P-190	Lung CA	50	30
P-019	Atypical Findings in Stomach	71	29
P-152	Lung CA	77	28
P-167	Pancreas	72	28
P-011	Lung CA	73	27
P-153	Pancreas	65	27
P-165	Lung CA	85	27
P-170	Duodenum	65	27
P-182	Barrett's Esophagus	58	27
P-146	Bile Duct	63	26
P-081	Barrett's Esophagus	74	26
P-151	Pancreas	49	25
P-155	Lung CA	60	25
P-156	Lung CA	57	25
P-150	Pancreas	78	23
P-149	Esophagus	59	19
P-154	Esophagus	80	19
P-169	Pancreas	71	19
P-168	Lung CA	63	18
P-180	Pancreas	67	13
P-144	Esophagus	59	9
P-147	Stomach	57	7
P-148	Stomach	69	6
P-171	Esophagus	76	0

As shown above, methods of the invention successfully screened 18 out of 27 patients who actually had non-colonic cancer. Only one patient was misdagnosed as having cancer when he did not. Thus, the methods of the invention are useful for non-invasive diagnosis of a non-specified cancerous disease state in a patient.

The threshold of 21 for a positive screen can be changed to accommodate desired sensitivities and specificities. For example, if 18 were the false negative results shown in Table 4 would be avoided. The skilled artisan knows how to set thresholds depending on the patient (e.g., a lower threshold for patients with symptoms than patients presenting with no symptoms), the disease being diagnosed, and the desired level of sensitivity and specificity. Regardless of the threshold, the principle of the invention remains that nucleic acid integrity is a viable marker for disease, and especially for cancer.

In addition, the propensity for disease may be measured using methods of the invention. For example, periodic molecular weight profiling in accordance with the methods of the invention may be used to monitor the disease state of a patient presenting no or minimal symptoms. Such longitudinal monitoring will determine whether a patient is progressing with increasing amounts of high integrity nucleic acids – indicating the desirability for follow-up examination.

What is claimed is:

1. A method for determining the disease status of a patient, the method comprising the steps of:
determining the integrity of nucleic acids in a patient sample comprising shed cells or cellular debris; and
identifying said patient as having disease if intact nucleic acids are present in said sample in an amount greater than a predetermined threshold.

2. A method for screening a patient for disease, the method comprising the steps of:
determining the integrity of nucleic acids in a patient sample comprising shed cellular debris; and
Identifying a positive screen as a sample in which the integrity of nucleic acids exceeds a predetermined threshold.

3. A method for detecting disease in a patient, the method comprising the steps of:
determining an amount of patient nucleic acid present in a patient sample;
comparing said amount with a standard amount of nucleic acid expected to be present in a disease-negative sample; and
detecting disease in said patient as an amount of patient nucleic acid greater than said standard amount.

4. The method of claim 3, wherein said nucleic acid is a fragment having a molecular weight of at least 1.3 Kb.

5. The method of claim 1, wherein said disease is cancer or pre-cancer.

6. The Method of claim 3, wherein said cancer is selected from the group consisting of colon cancer, lung cancer, esophageal cancer, prostate cancer, stomach cancer, pancreatic cancer, liver cancer, and lymphoma.

7. The method of claim 1, wherein said nucleic acid is selected from the group consisting of K-ras, p53, apc, dcc, tumor suppressor genes, and oncogenes.

8. The method of claim 1, wherein said patient sample is selected from the group consisting of stool, sputum, pancreatic fluid, bile, lymph, blood, urine, cerebrospinal fluid, seminal fluid, saliva, breast nipple aspirate, and pus.

9. A method for screening a patient sample to determine the disease status of a patient, the method comprising the steps of:

(a) amplifying patient nucleic acid in a sample obtained from a patient;

(b) determining an amount of amplified nucleic acid obtained in Step (a); and

(c) identifying a positive screen when said amount of amplified nucleic acid is greater than an amount of amplified nucleic acid expected in a disease-negative sample.

10. A method for screening a biological sample for disease, the method comprising the steps of:

selecting a plurality of genomic loci;

conducting an amplification reaction at each of said loci;

identifying a positive screen as a sample in which the number of loci producing an amplification product exceeds a predetermined threshold.

11. A method for screening a patient sample for disease, the method comprising the steps of:

selecting a plurality of genomic loci;

conducting an amplification reaction at each of said loci;

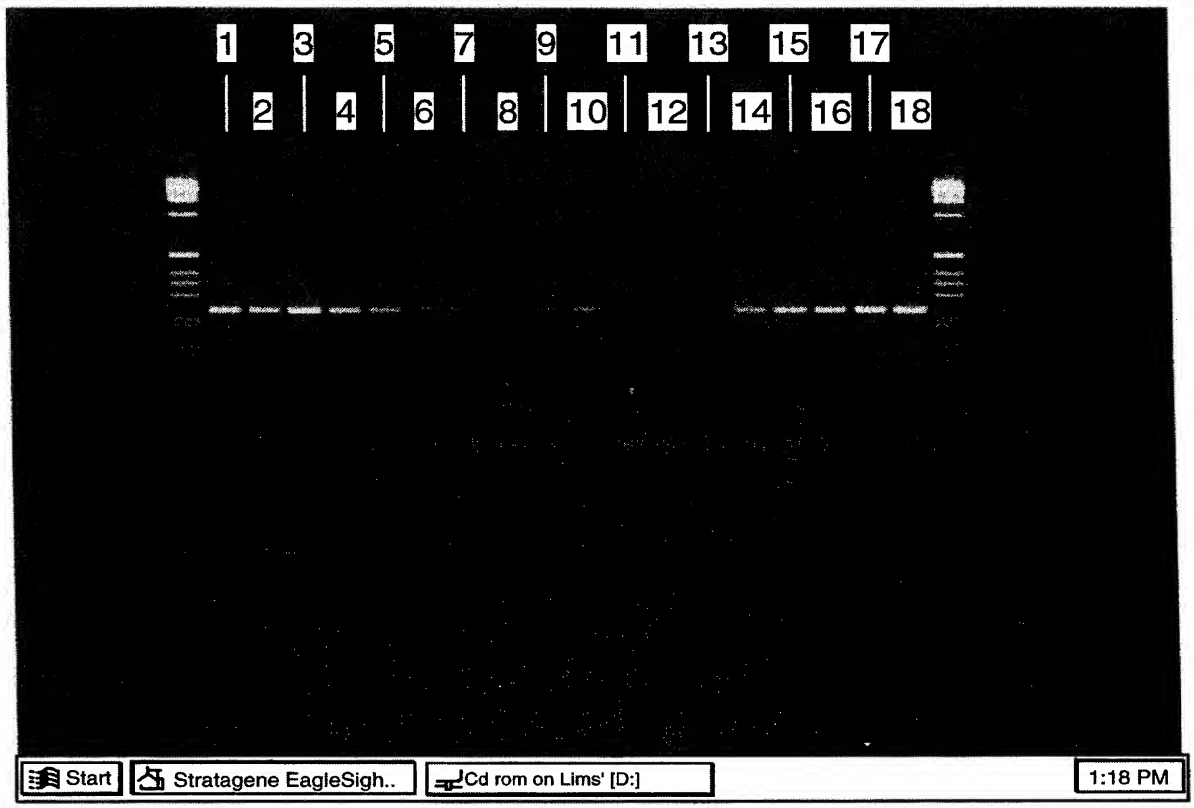
assigning a first numerical score to each locus in which amplification takes place;

assigning a second numerical score to each locus in which amplification does not take place;

determining whether a total of said first numerical score exceeds a total of said second numerical score by a threshold amount, thereby to screen said sample for disease.

12. A nucleic acid fragment isolated from a patient sample, said nucleic acid being at least 1.3 Kb and indicative of nucleic acid integrity associated with cancer.

13. An isolated nucleic acid obtained by the method of claim 1.

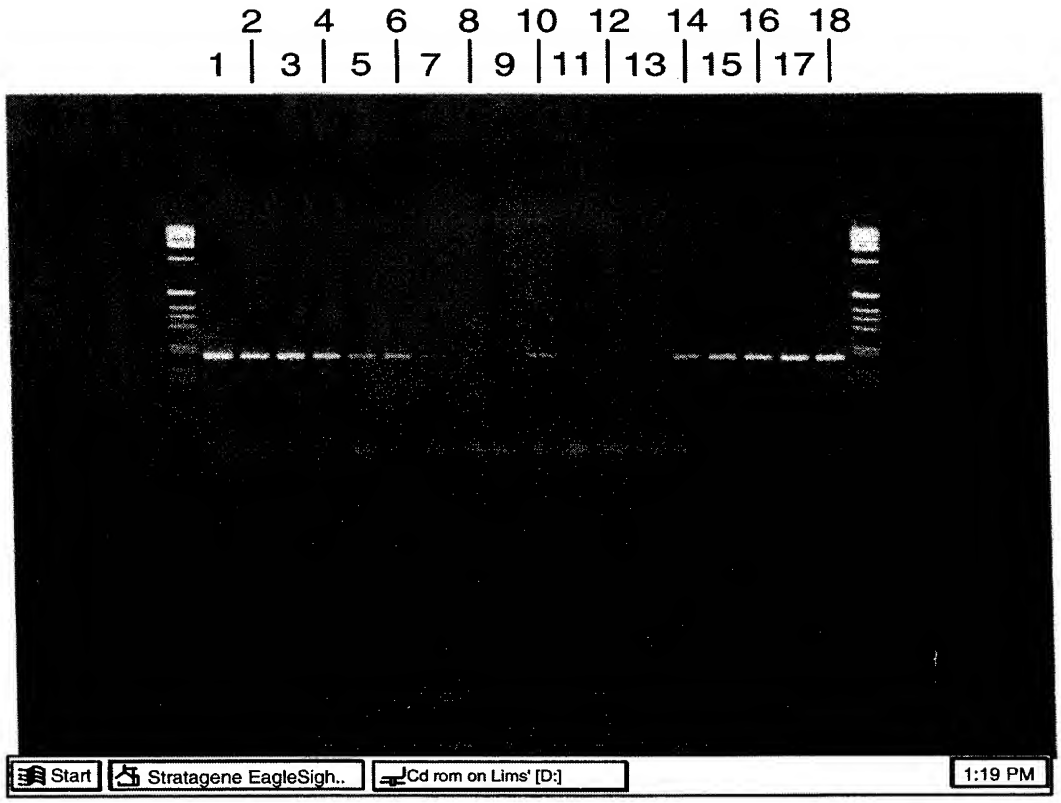


200bp
AMPLIFICATIONS
33 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	7903.8	ABNORMAL	1	A
2	5627.4	ABNORMAL	2	A
3	8809.11	ABNORMAL	3	A
4	5421.94	ABNORMAL	4	A
5	1838.07	POSITIVE CONTROL		B
6	-549.23	NORMAL	5	C
7	-715	NORMAL	6	C
8	-1605.13	NORMAL	7	C
9	-824.73	NORMAL	8	C
10	259.77	NORMAL	9	C
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >2000
B= 500-2000
C= <500

FIG. 1

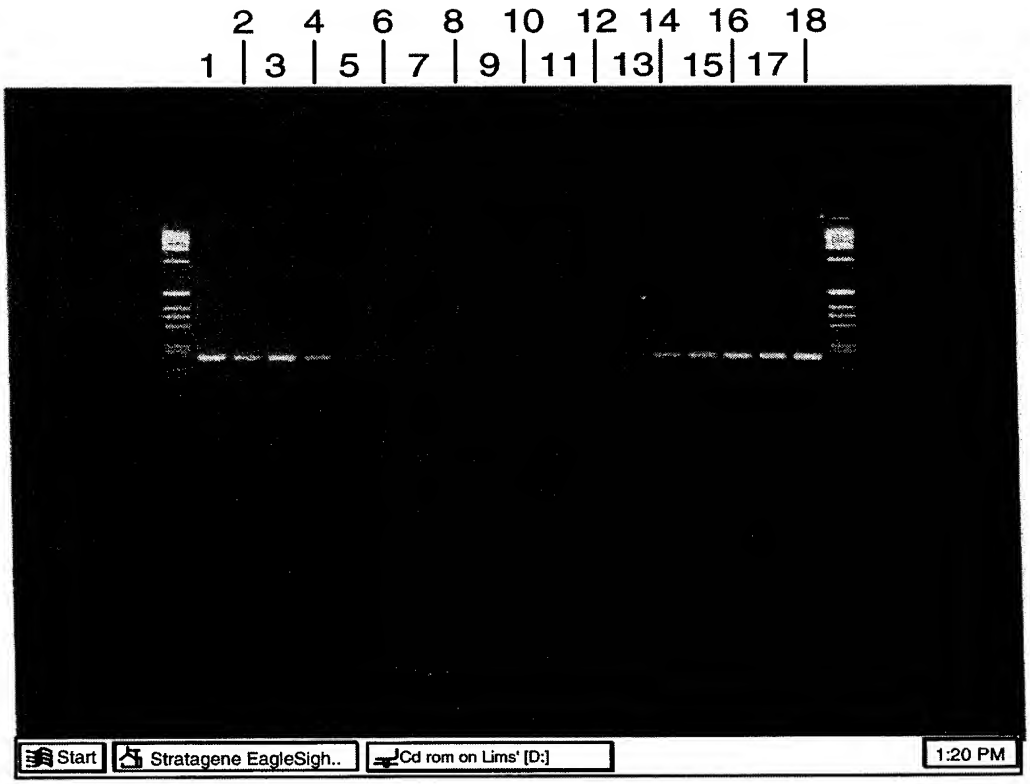


200bp
AMPLIFICATIONS
35 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	10851.04	ABNORMAL	1	A
2	8862.34	ABNORMAL	2	A
3	9777.85	ABNORMAL	3	A
4	6874.28	ABNORMAL	4	A
5	2392.07	POSITIVE CONTROL		B
6	3080.62	NORMAL	5	B
7	813.45	NORMAL	6	C
8	-720.04	NORMAL	7	C
9	-442.2	NORMAL	8	C
10	1353.86	NORMAL	9	B
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >5000
B= 1000-5000
C= <1000

FIG. 2

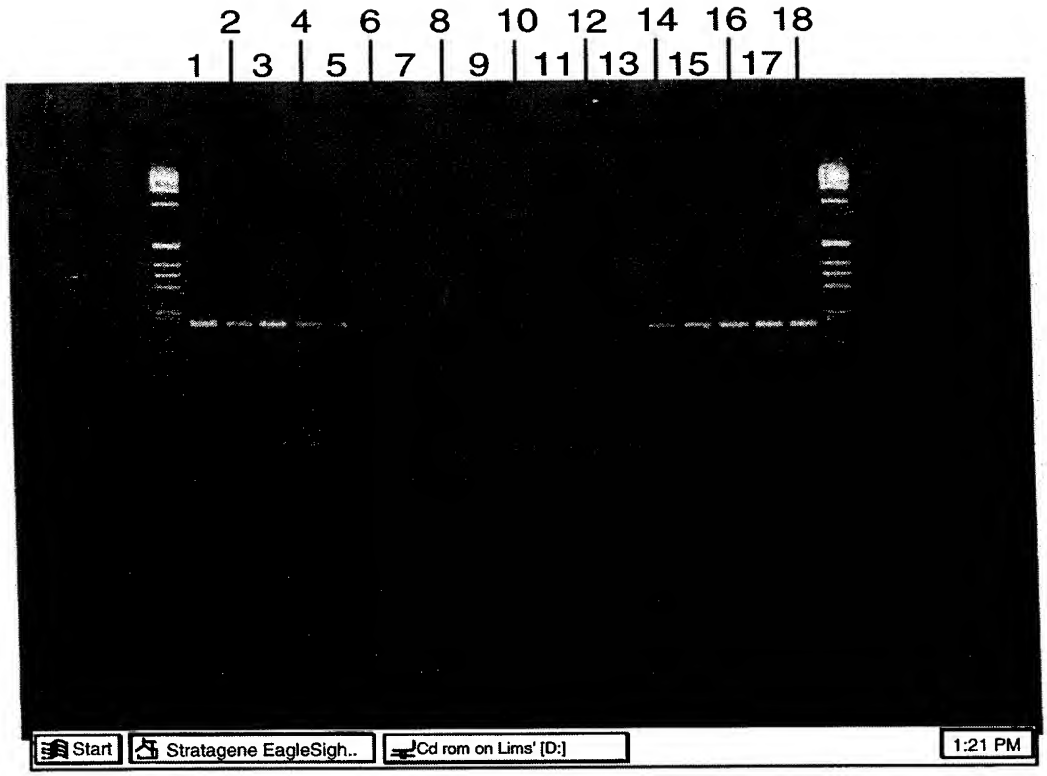


200bp
AMPLIFICATIONS
34 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	8428.34	ABNORMAL	1	A
2	4917.31	ABNORMAL	2	A
3	7742.22	ABNORMAL	3	A
4	3049.85	ABNORMAL	4	A
5	409.5	POSITIVE CONTROL		B
6	-682.75	NORMAL	5	C
7	-781.09	NORMAL	6	C
8	-1099.28	NORMAL	7	C
9	-1015.39	NORMAL	8	C
10	359.74	NORMAL	9	B
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >750
B= 250-750
C= <250

FIG. 3

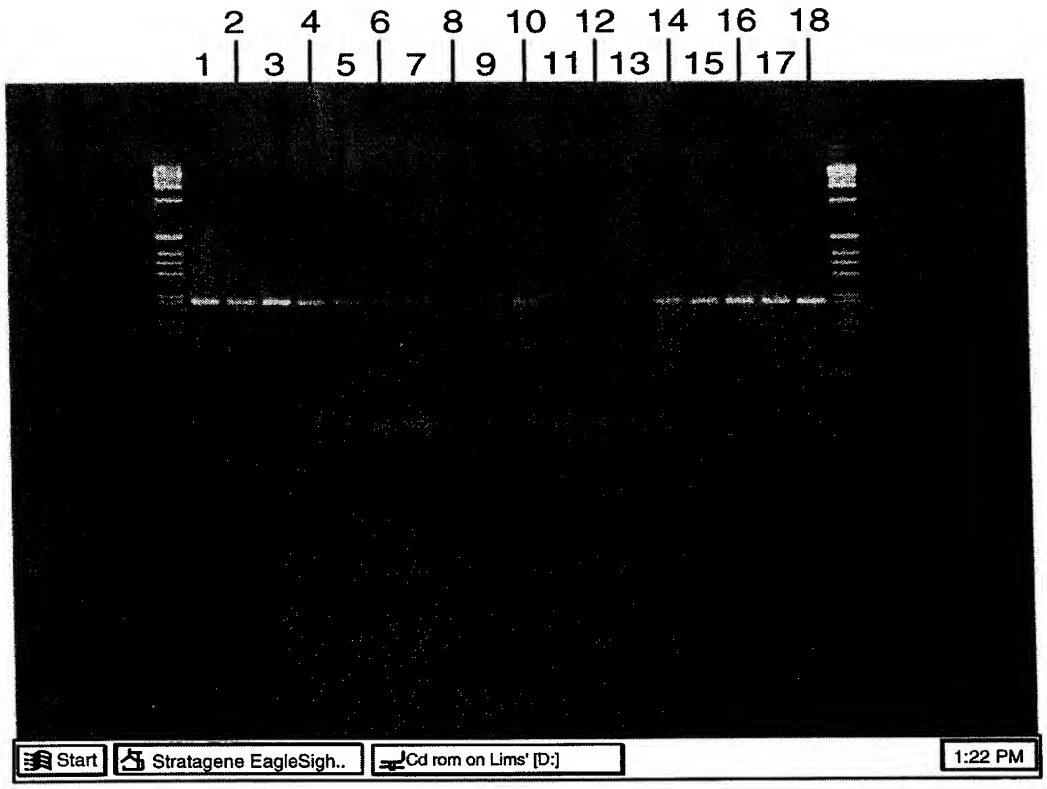


200bp
AMPLIFICATIONS
33 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	7879.15	ABNORMAL	1	A
2	4079.09	ABNORMAL	2	A
3	7995.95	ABNORMAL	3	A
4	2600.3	ABNORMAL	4	A
5	1698.19	POSITIVE CONTROL		B
6	-405.32	NORMAL	5	C
7	-466.15	NORMAL	6	C
8	-1046.47	NORMAL	7	C
9	-764.83	NORMAL	8	C
10	105.05	NORMAL	9	C
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >2000
B= 500-2000
C= <500

FIG. 4

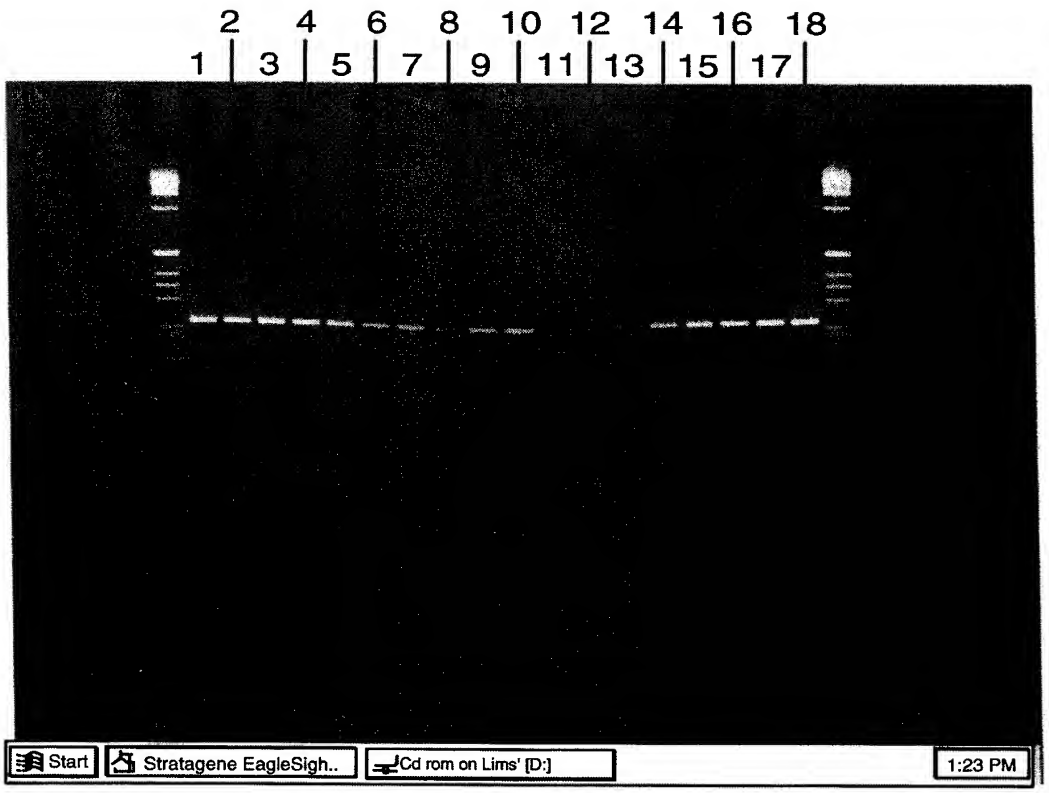


200bp
AMPLIFICATIONS
34 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	7852.95	ABNORMAL	1	A
2	4797.07	ABNORMAL	2	A
3	8543.47	ABNORMAL	3	A
4	3597.23	ABNORMAL	4	A
5	943.84	POSITIVE CONTROL		B
6	-296.7	NORMAL	5	C
7	-5.48	NORMAL	6	C
8	-896.94	NORMAL	7	C
9	-196.87	NORMAL	8	C
10	414.81	NORMAL	9	C
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >2000
B= 500-2000
C= <500

FIG. 5

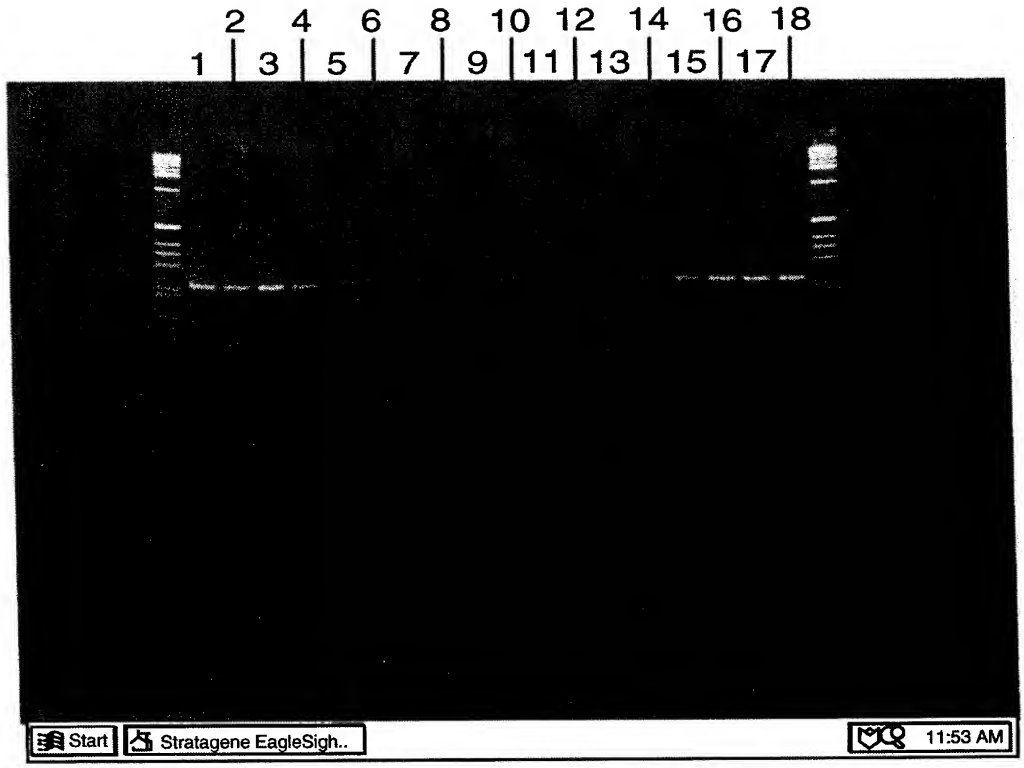


200bp
AMPLIFICATIONS
34 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	7660.6	ABNORMAL	1	A
2	7032.89	ABNORMAL	2	A
3	8364.31	ABNORMAL	3	A
4	6892.04	ABNORMAL	4	A
5	4883.47	POSITIVE CONTROL		A
6	1934.67	NORMAL	5	B
7	1380.84	NORMAL	6	B
8	-964.17	NORMAL	7	C
9	1729.51	NORMAL	8	B
10	2221.69	NORMAL	9	B
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >5000
B= 1000-5000
C= <1000

FIG. 6



200bp
AMPLIFICATIONS
33 CYCLES

LANE	Q#	SAMPLE TYPE	SAMPLE NUMBER	GRADE
1	8519.13	ABNORMAL	1	A
2	5745.19	ABNORMAL	2	A
3	9765.65	ABNORMAL	3	A
4	4153.79	ABNORMAL	4	A
5	1869.33	POSITIVE CONTROL		B
6	418.37	NORMAL	5	C
7	405.91	NORMAL	6	C
8	-258.08	NORMAL	7	C
9	141.64	NORMAL	8	C
10	450.78	NORMAL	9	C
11		NEG CONTROL	-	
12		NEG CONTROL	-	
13	400	400	STANDARD	
14	2000	2000	STANDARD	
15	4000	4000	STANDARD	
16	6000	6000	STANDARD	
17	8000	8000	STANDARD	
18	10000	10000	STANDARD	

A= >2000
B= 500-2000
C= <500

FIG. 7

1.8kb
AMPLIFICATIONS
36 CYCLES

LANE	Q#	SAMPLE
1		NEG CONTROL
2	102.935	ABNORMAL
3	260.645	ABNORMAL
4	0.075	NORMAL
5	48.305	ABNORMAL
6	0.045	NORMAL
7	18.575	NORMAL
8		NEG CONTROL
9		NEG CONTROL
10	75	75
11	125	125
12	250	250
13	500	500
14	1000	1000

ABNORMAL / NORMAL CUTOFF 40

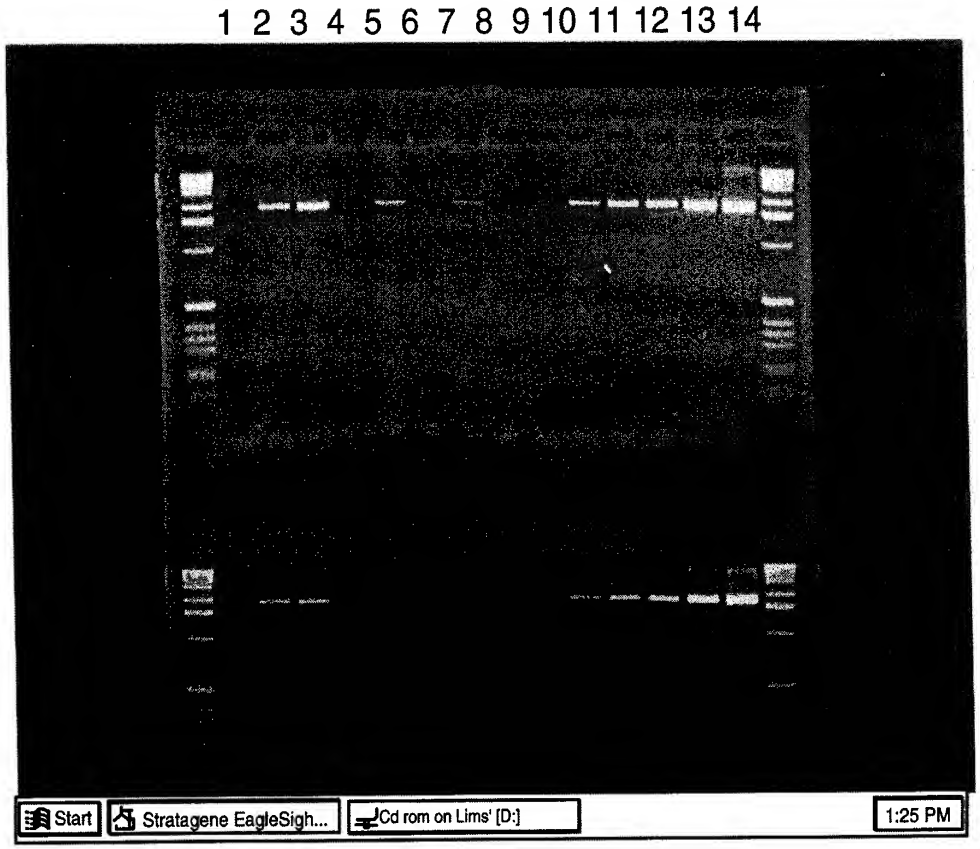


FIG. 8

1.8kb
AMPLIFICATIONS
38 CYCLES

LANE	Q#	SAMPLE
1		NEG CONTROL
2	81.84	ABNORMAL
3	91.515	ABNORMAL
4	0.04	NORMAL
5	24.86	ABNORMAL
6	0.88	NORMAL
7	17.25	NORMAL
8		NEG CONTROL
9		NEG CONTROL
10	75	75
11	125	125
12	250	250
13	500	500
14	1000	1000

ABNORMAL / NORMAL CUTOFF 20

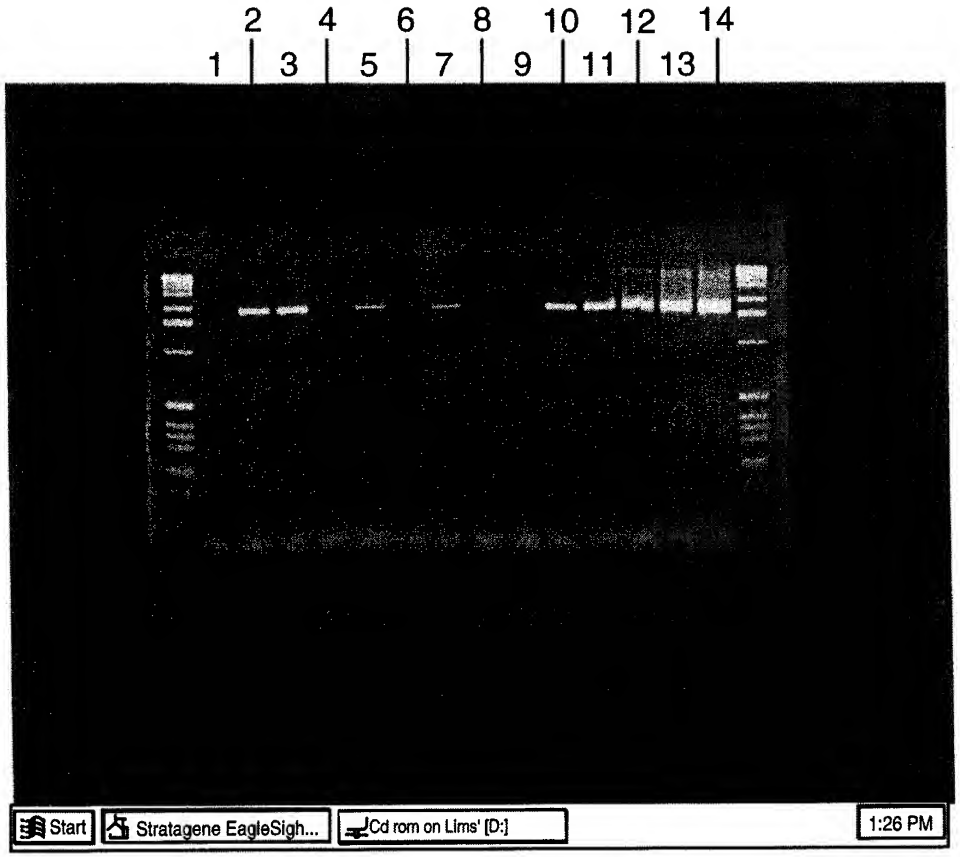


FIG. 9

1.8kb
AMPLIFICATIONS
40 CYCLES

LANE	Q#	SAMPLE
1		NEG CONTROL
2	70.72	ABNORMAL
3	92.78	ABNORMAL
4	96.76	ABNORMAL
5	0.00	NORMAL
6	29.85	ABNORMAL
7	0.00	NORMAL
8	2.00	NORMAL
9		NEG CONTROL
10		NEG CONTROL
11	75	75
12	125	125
13	250	250
14	500	500
15	1000	1000
16	2000	2000

ABNORMAL / NORMAL CUTOFF 10

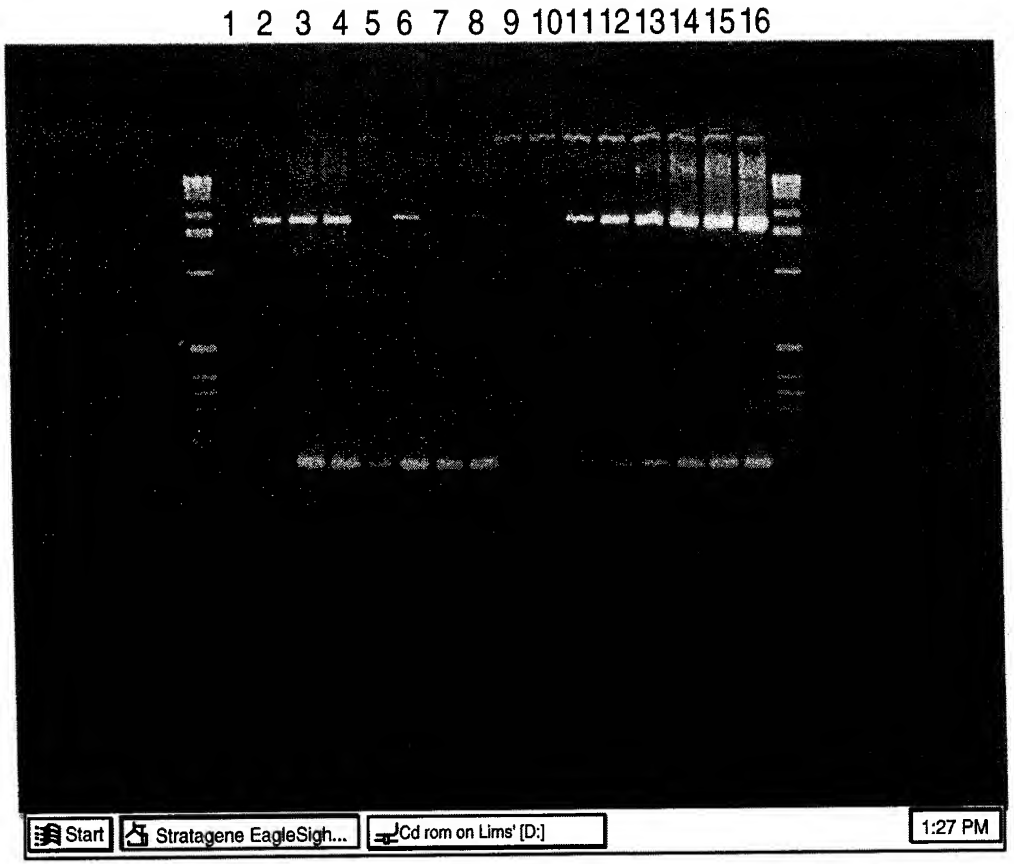


FIG. 10

GEL #1

LANE #	CLINICAL STATUS
A	MARKER LANE
N	NEGATIVE CONTROL
N	NEGATIVE CONTROL
1	CANCER
2	NORMAL
3	CANCER
4	NORMAL
5	NORMAL
6	NORMAL
7	NORMAL
8	NORMAL
9	NORMAL
10	NORMAL
11	CANCER
12	NORMAL
13	NORMAL
14	NORMAL
15	NORMAL
N	NEGATIVE CONTROL
NA	STANDARD CURVE
NA	STANDARD CURVE
NA	STANDARD CURVE
NA	STANDARD CURVE
NA	STANDARD CURVE
B	MARKERS

RESULTS

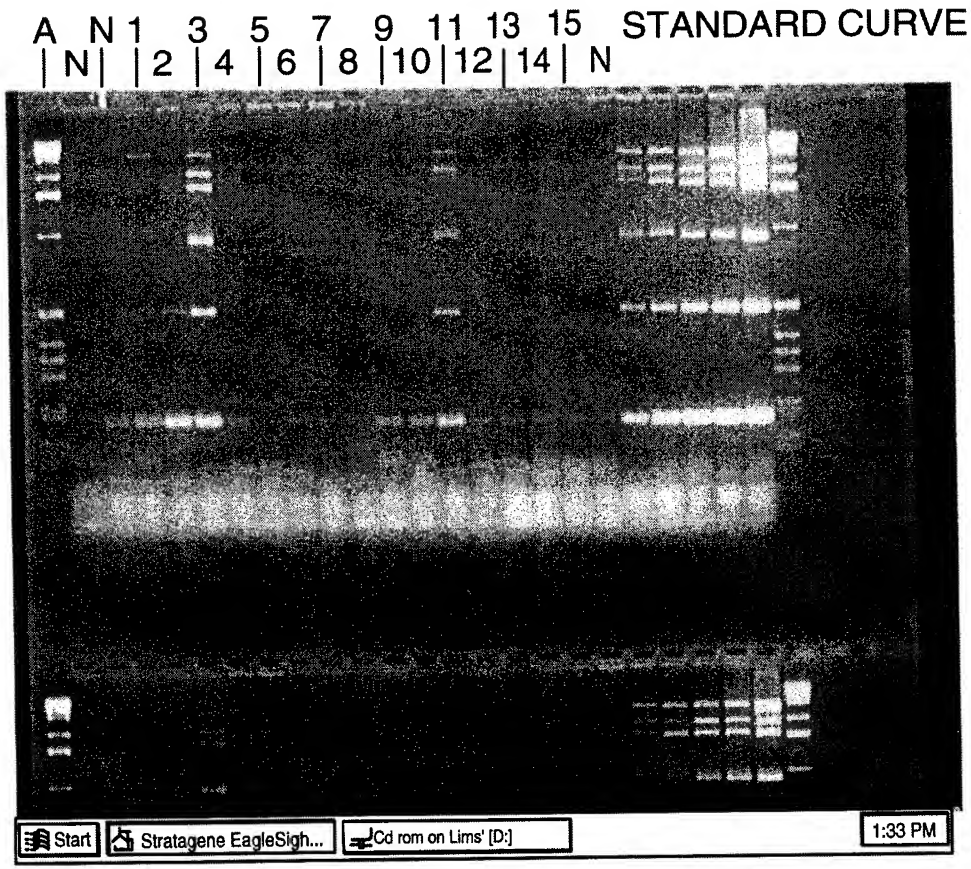


FIG. 11A

GEL #2

LANE #	CLINICAL STATUS
A	MARKERS
N	NEGATIVE CONTROL
N	NEGATIVE CONTROL
16	NORMAL
17	NORMAL
18	CANCER
19	NORMAL
20	NORMAL
21	NORMAL
22	NORMAL
23	NORMAL
24	NORMAL
25	NORMAL
26	NORMAL
27	NORMAL
28	NORMAL
29	NORMAL
30	NORMAL
N	NEGATIVE CONTROL
NA	STANDARD CURVE
NA	STANDARD CURVE
NA	STANDARD CURVE
NA	STANDARD CURVE
NA	STANDARD CURVE
B	MARKERS

RESULTS

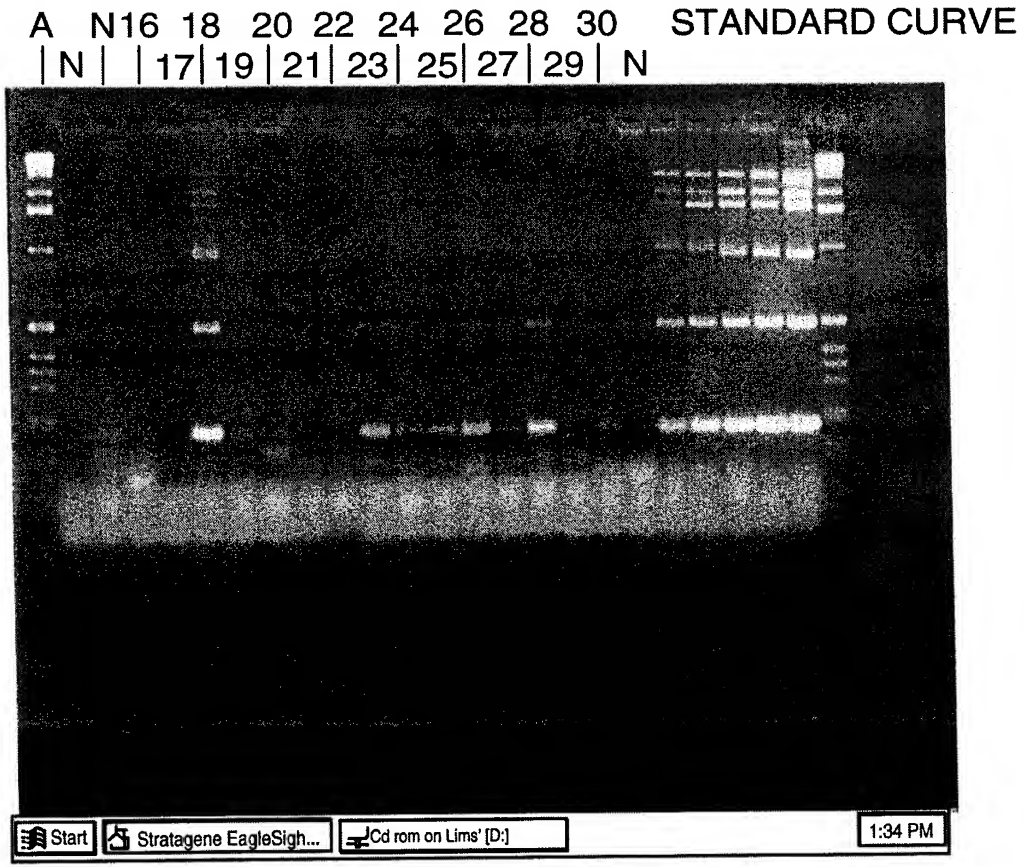


FIG. 11B

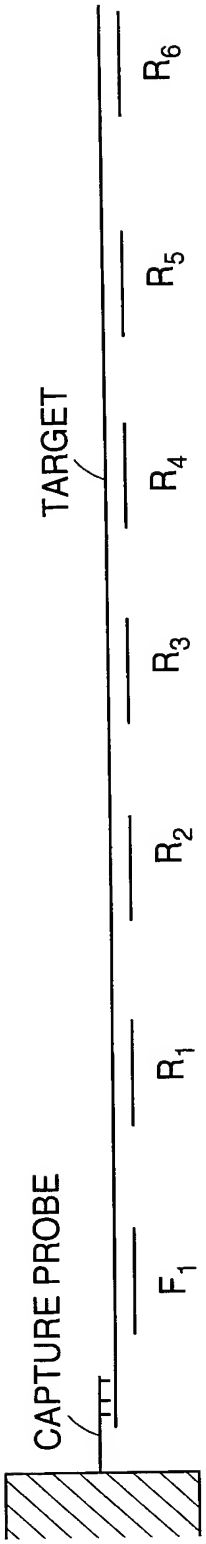

CAPTURE PROBE
F1
R1
R2
R3
TARGET
R4
R5
R6

FIG. 12

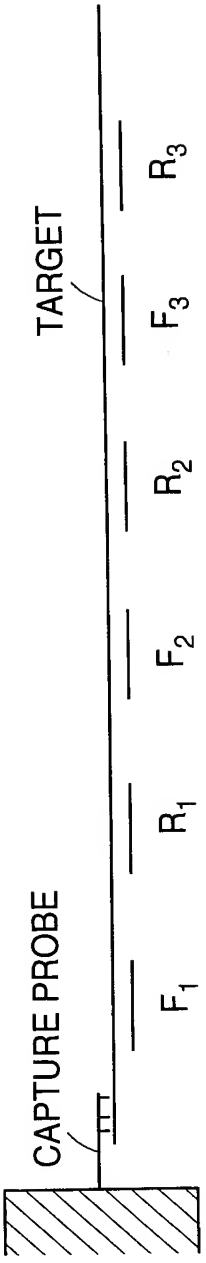

CAPTURE PROBE
F1
R1
F2
R2
TARGET
F3
R3

FIG. 13

SEQUENCE LISTING

<110> Shuber, Anthony P

<120> Methods for Disease Detection

<130> EXT-034PC

<140>
<141>

<150> US 60/152,847
<151> 1999-09-08

<150> US 09/455,950
<151> 1999-12-7

<160> 11

<170> PatentIn Ver. 2.0

<210> 1
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:K-ras probe

<400> 1
gtggagtatt tgatagtgta ttaaccttat gtgtgac 37

<210> 2
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:apc probe
apc-1309

<400> 2
ttccagcagt gtcacagcac cctagaacca aatccag 37

<210> 3
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:apc probe
apc-1378

<400> 3
cagatagccc tggacaaaca atgccacgaa gcagaag 37

<210> 4
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:p53 exon 5 probe

<400> 4
tactcccctg ccctcaacaa gatgttttgc caactgg 37

<210> 5
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:p53 exon 7 probe

<400> 5
atttcttcca tactactacc catcgacctc tcatc 35

<210> 6
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:p53 exon 7 probe

<400> 6
atgaggccag tgcgccttgg ggagacctgt ggcaagc 37

<210> 7
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:p53 exon 8 probe

<400> 7
gaaaggacaa gggtggttgg gagtagatgg agcctgg 37

<210> 8
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:BRCA1 probe

<400> 8
gattctgaag aaccaacttt gtccttaact agctctt 37

<210> 9
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:BRCA2 probe

<400> 9
ctaagtttga atccatgctt tgctcttctt gattatt 37

<210> 10
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:APC1 probe

<400> 10
cagatagccc tggacaaacc atgccaccaa gcagaag 37

<210> 11
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:APC2 probe

<400> 11
gaagttcctg gattttctgt tgctggatgg tagttgc 37